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14. ABSTRACT

The overall purpose of my project is to investigate the direct interaction of ER-alpha with eNOS and nitrite, and to determine what roles they might play in hormone independent breast cancers. I hope to characterize the interaction between ER-alpha and eNOS in the absence and presence of growth factors, determine whether polymorphic forms of eNOS increase ER-alpha activity due to increased nitrite production, and to determine the mechanism by which nitrite activates ER-alpha. Important findings to date have shown that treatment of wild-type ER-alpha with nitrite leads to the dissociation of the hsp90 complex, binding to DNA, and the recruitment of the coactivator SRC-1 and RNA polymerase II to estrogen regulated gene promoters. These findings provide a novel role for nitrite in activation of ER-alpha.

15. SUBJECT TERMS

estrogen receptor-alpha, anions, nitric oxide synthase, nitrite, mechanisms, metalloestrogens

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Introduction:

Our lab has shown that treatment MCF-7 breast cancer cells with growth factors leads to the activation of Akt, and the subsequent activation of endothelial nitric oxide synthase (eNOS) (Figure 1). Treatment of MCF-7 cells with IGF-1 led to an increase in the expression of estrogen regulated genes and was blocked by an eNOS inhibitor and the anti-estrogen ICI 182,720. Further testing enabled us to establish that nitrite, the major breakdown product of nitric oxide in the cell, was mediating the effects of the Akt/eNOS pathway in MCF-7 cells by activation of estrogen receptor-alpha (ER-alpha). In addition, mutational analysis of ER-alpha revealed six amino acids within the ligand binding domain of the receptor that are important in the activation of ER-alpha by nitrite (1). Determining the mechanism by which nitrite activates ER-alpha will provide valuable information in future treatment of breast cancer. The overall goal of my project is to investigate the interaction of ER-alpha with eNOS and nitrite, and what role they might play in hormone independent breast cancers.

Body:

Objective 1: To characterize the physiological interaction of ER-alpha with eNOS (*18 months*).

Antibodies for ER-alpha and eNOS have been selected. Protocol for coimmunoprecipitation has been developed, although objective 1 has not been completed. I would also like to acquire a suitable positive control to determine the level of eNOS present in MCF-7 cells.

Objective 2: Determine whether polymorphic forms of eNOS increase the activity of ER-alpha due to increased synthesis of nitrite (*6 months*).

To date our lab has not been able to obtain any polymorphic forms of eNOS in order to test their ability to increase nitrite levels and ER-alpha activity.

Recently, we have been able to determine nitrite levels in MCF-7 cells in the presence and absence of IGF-1 and the eNOS inhibitor N^G-nitro-L-arginine methyl ester HCl (L-NAME) using a nitrate/nitrite detection kit. Briefly, MCF-7 cells were routinely passaged in IMEM with phenol red containing 5% fetal bovine serum. One day before growth factor treatment the tissue

culture media was replaced with phenol red free IMEM and 5% charcoal stripped FBS, immediately before treatment the tissue culture media was changed a final time to phenol red free IMEM with 1% stripped serum. Following a 16 hour treatment of MCF-7 cells with 40ng/ml IGF-1, total nitrite/nitrate levels in the tissue culture media increased to an average of 74.5 umol/L compared to control level of 25.8 umol/L. Upon adding the inhibitor of eNOS (L-NAME) in combination with IGF-1 treatment, total nitrite/nitrate concentration within the media decreased to 27.1 umol/L (Figure 2). Therefore, in MCF-7 cells the growth factor IGF-1 is able to increase total nitrite/nitrate production in the cell and this effect is blocked by L-NAME.

Objective 3. Determine the mechanism by which nitrite activates the estrogen receptor-alpha *(36 months)*.

Our lab has shown that nitrite is able to interact with specific amino acids located on helices H4, H10, H11, and H12 of the ligand-binding domain of the receptor. Molecular modeling suggest that lys529 on H11 and asn532 on the loop between H11 and H12 form the first site of interaction, his516 on H10 and lys520 on H11 form a second, and cys381 on H4 and his547 on H12 form the final site of interaction (Figure 3). I predict that the interaction of nitrite with site 1 (S1) is necessary for the initial movement of helix H12 over the ligand-binding pocket of the receptor allowing for disassociation from the hsp90 complex. Therefore, mutants of S1 will remain associated with hsp90 upon treatment with nitrite. Site 2 (S2) is involved in the straightening of helices H10 and H11, creating a one continuous helix. This helix makes up a portion of the dimerization domain of the receptor and therefore, S2 mutants should dissociate from the hsp90 complex. Finally, the last interaction site is necessary for the proper formation of the coactivator binding site. A shallow hydrophobic groove formed by the positioning of helix H12 under helix H4 and the movement of helix H3 into the proper alignment is necessary for coactivator binding to the receptor. Therefore, site 3 (S3) mutants should dissociate from the hsp90 complex, bind DNA, but not recruit coactivator necessary for transcription. I have tested the ability of wild type and mutant forms of ER-alpha to dissociate from the hsp90 complex, translocate into the nucleus and bind DNA, recruit SRC-1 and RNA polymerase II to estrogen regulated gene promoters.

In order to test the ability of nitrite to disrupt the hsp90-receptor complex coimmunoprecipitation assays were performed in HEK 293 cells. Briefly, wild type and mutant forms of ER-alpha were transfected into HEK 293 cells, 48 hours post-transfection the cells were

either left untreated or treated with 1nM estradiol or 1uM sodium nitrite for three hours. Following treatment the cells were collected, lysed, incubated with antibody to ER-alpha (H-184, Santa Cruz) and immunoblot was performed with antibody to hsp90. All forms of receptor dissociated hsp90 upon treatment with estradiol as expected (Figures 4 and 5). However, upon treatment with nitrite, the mutant lys529 and asn532 failed to dissociate from hsp90 (Figure 5). As an additional control wild-type ER-alpha was tested for its ability to dissociate from hsp70, which is another protein in the inactive receptor complex, and hsp70 dissociated upon treatment with estradiol and nitrite (Figure 4). Based upon these results lys529 and asn532 appears to be critical for the disruption of the hsp90-receptor complex upon activation by nitrite.

In order to test the ability of nitrite to activate the receptor and allow it to be recruited to DNA, chromatin immunoprecipitation (ChIP) assays were conducted in HEK 293 cells transfected with ER-alpha. A region of the human complement C3 (hC3) promoter and pS2 promoters containing estrogen response elements (EREs) were amplified by polymerase chain reaction (PCR) using promoter specific primers. Following a three hour treatment with either estradiol or nitrite, wild type and mutant expressing HEK 293 cells were cross-linked with 1% formaldehyde, collected and lysed. Cell lysates were precipitated over night with antibody for ER-alpha (H-184, Santa Cruz), washed, crosslinks were reverse and PCR was preformed. Upon treatment with estradiol, all forms of ER-alpha were recruited to both the hC3 and pS2 promoters (Figure 6, 7, and 9). However, upon nitrite treatment only the wild type, lys520 and his547 forms of the receptor were recruited to the promoters (Figure 6, 7, and 9). In addition to the results of ChIP assays using standard PCR, I have also conducted quantitative PCR experiments using SYBR green in order to determine the relative amount of immunoprecipitated DNA for all forms of ER-alpha treated with either estradiol or nitrite. Results obtained using this method are comparable to those using the standard PCR method. Upon treatment with estradiol, most forms of the receptor were recruited to the hC3 promoter between 3 and 4-fold over control treated samples, with lys520 having been recruited slightly higher (Figure 8). Further, only wild-type, lys520 and his547 were recruited to the hC3 promoter in the presence of nitrite as seen in previous results (Figure 8 and 10). Similar results were obtained for the pS2 promoter, with receptor being recruited 3 to 4-fold above control, and higher levels for lys520 and lys529 (Figure 10). Further, wild-type, lys520 and his547 were recruited to the pS2 promoter in the presence of nitrite (Figure 10).

The final task for objective three will be to investigate the interaction of wild type and mutant ER-alphas with coactivators and RNA polymerase II after treatment with nitrite. Re-ChIP assays have been conducted in order to determine if the steroid receptor coactivator SRC-1 and RNA polymerase II have been recruited to the promoter by ER-alpha. Re-ChIP assays were performed as described above for regular ChIP, however, following the first step of immunoprecipitation with antibody to ER-alpha, the captured chromatin was washed and mildly dissociated and then immunoprecipitated a second time with either an antibody to SRC-1 (C-20, Santa Cruz) or with antibody to RNA pol II (CTD4H8, Millipore). The purified DNA was subjected to PCR analysis. Estradiol treatment recruited both SRC-1 and RNA pol II to the hC3 and pS2 promoters for all mutants, while nitrite only recruited SRC-1 and RNA pol II in the presence of the wild-type receptor (Figures 11-16). Currently, I am analyzing the results from quantitative PCR performed on samples from re-ChIP experiments using both SRC-1 and PolII.

In addition, I am currently measuring mRNA levels of both hC3 and pS2 following treatment with estradiol and nitrite for each form of the receptor.

Key Research Accomplishments:

- Transfection
- ChIP assays
- Re-ChIP assay
- Nitrate/Nitrite assay
- Co-immunoprecipitation
- Immunoblot
- qPCR
- RNA preparation
- Reverse transcription reaction

Reportable Outcomes:

- Poster Presentation Endocrine Society June 2010, San Diego
- Poster Presentation Lombardi Research Days April 2011, Lombardi Comprehensive Cancer Center, Georgetown University

Conclusions

The work outlined in this report to date has shown that upon treatment with 1uM nitrite, wild-type ER-alpha dissociates from the hs90 complex, binds to DNA, and recruits SRC-1 and RNA polymerase II to estrogen regulated genes similar to estradiol. Site A mutants lys529 and asn532 do not dissociate from the hsp90 complex, bind DNA and coactivator. Site B mutants, his516 and lys520 dissociate from the hsp90 complex; however, his516 did not bind DNA, while lys520 did. Finally, site C mutants dissociated from the hsp90 complex, cys381 did not bind to DNA, while his547 was recruited to DNA but did not bind coactivator. In addition, treatment of MCF-7 cells with the growth factor IGF-1 leads to an increase in nitrite/nitrate produced by the cell and blocked by the addition of the eNOS inhibitor L-NAME. Future investigations will begin to characterize the interaction between eNOS and ER-alpha and what effect polymorphic forms of eNOS might have on breast cancer progression. In addition, our lab is hoping to begin work on determining what role eNOS and nitrite might play in the growth of estrogen independent breast cancer cells lines developed here at the Lombardi Cancer Center.

References

1. D.J. Veselik, S. Divekar, S. Dakshanamurthy, G.B. Storchan, J.M. Turner, K.L. Graham, L. Huang, A. Stoica and M.B. Martin, Activation of estrogen receptor-alpha by the anion nitrite, *Cancer Res.* **68** (2008), pp. 3950–3958

Appendices

Endocrine Society June 2010 Accepted Abstract Preview

Title: Activation of Estrogen Receptor-α by Nitrite

GB Storchan, MS, gbs7@georgetown.edu[1], KL Koenig klk38@georgetown.edu[1] and MB Martin, PhD, martinmb@georgetown.edu[1]. [1]Biochemistry and Molecular & Cellular Biology and Oncology, Georgetown University, Washington, DC, United States,20007.

Body: Recent studies show that the anion nitrite binds to and activates estrogen receptor-alpha (ER-alpha). Mutational analysis and molecular modeling identified three potential nitrite binding sites in the ligand binding domain (LBD) of the receptor. Site A is formed by lys529 on helix H11 and asn532 in the loop between helices H11 and H12; site B is formed by his516 on helix H10 and lys520 on helix H11; and site C is formed by cys381 on helix H4 and his547 on helix H12. To determine the mechanism by which nitrite activates the receptor, wild-type and mutants forms of ER-alpha were tested for their ability to dissociate from the heat shock protein 90 (hsp90) complex, translocate into the nucleus, dimerize and bind DNA, interact with coactivator, and recruit SRC-1 and RNA polymerase II. Preliminary results demonstrate that, upon treatment with 1uM nitrite, wild-type ER-alpha dissociated from hsp90, was recruited to DNA, bound coactivator, and recruited SRC-1 and RNA polymerase II to DNA. Site A mutants, K529A and N532A, did not dissociate from the hsp90-receptor complex, bind to DNA or bind coactivator. The site B mutants, H516A and K520A, dissociated from hsp90. However, H516A did not bind to DNA or recruit coactivator, while K520A bound to DNA, but did not recruit coactivator. The site C mutants, C381A and H547A, dissociated from hsp90. C381A failed to bind to DNA, while H547A was recruited to DNA but failed to bind coactivator. The results suggest a model whereby the interaction of nitrite with site A results in a conformational change at the interface of helix H11 and loop 11-12 that is necessary for the dissociation of hsp90. Site B is involved in the formation of a continuous helix between helices H10 and H11 that is necessary for binding to DNA. Site C, formed by amino acids located on helices H4 and H12, is involved in the recruitment to DNA as well as the formation of the coactivator binding site. Together, these findings represent a novel role for the anion nitrite in the activation of ER-alpha.

Sources of Support: National Institutes of Health R21ES015160-02 awarded to MBM DOD BCRP Predoctoral Traineeship Award BC073599 awarded to GBS

Supporting Data

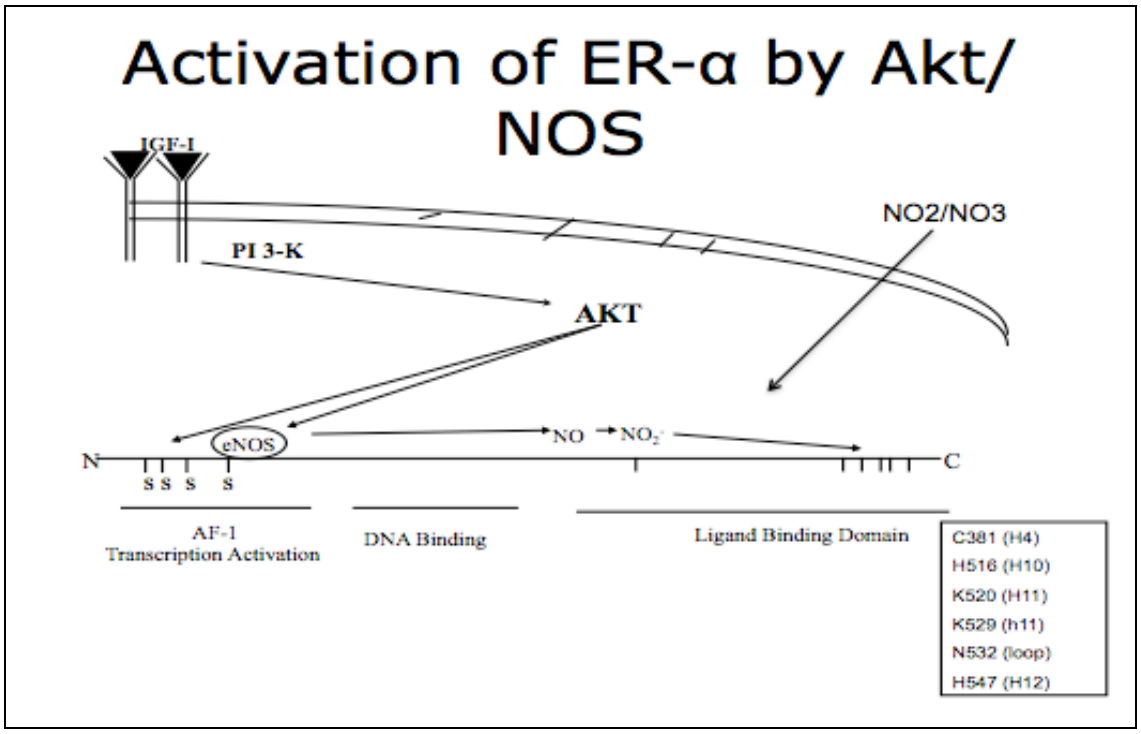

Figure 1. Activation of ER-alpha by eNOS through the Akt pathway. Activation of Akt through the IGF-1 pathways, leads to the phosphorylation and activation of eNOS. eNOS increases the production into the cell. Nitrite is then able to interact with amino acids located within the ligand-binding domain of ER-alpha of nitric oxide, which is rapidly converted to nitrite

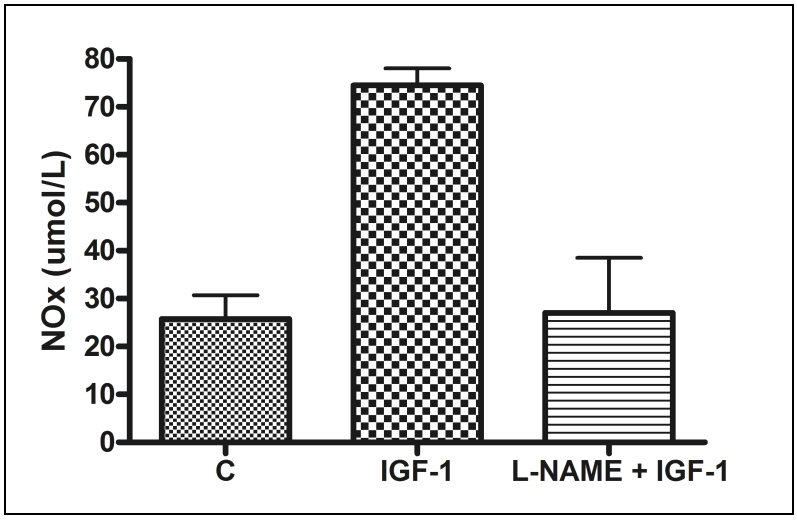

Figure 2. Effect of IGF-1 on total nitrite/nitrate production in MCF-7 cells. MCF-7 breast cancer cells were treated with either 40 ng/mL IGF-1, 40 ng/mL IGF-1 and 0.5mM L-NAME or untreated control for 16 hours. Cell culture supernatant was collected and used to determine total nitrite/nitrate.

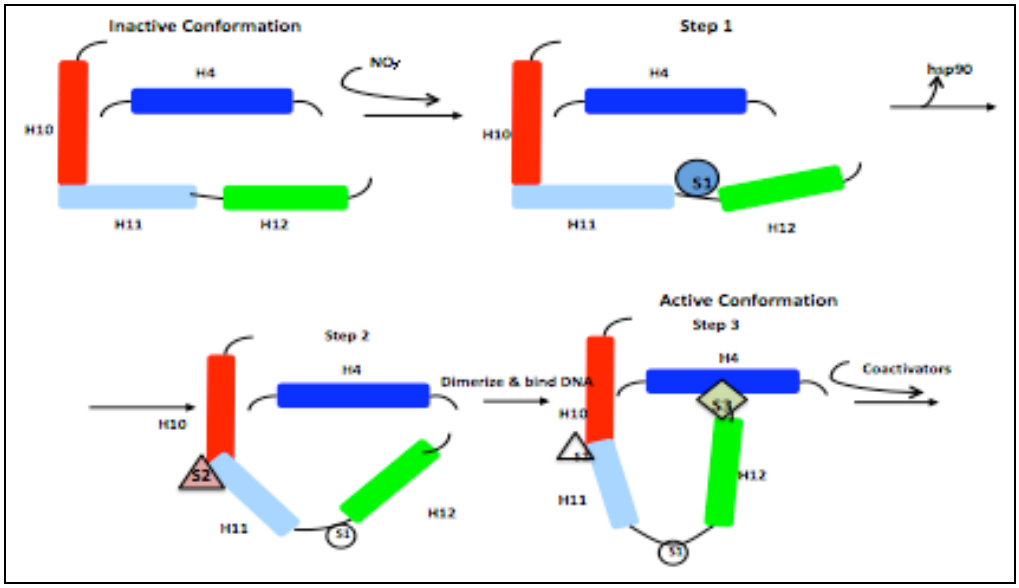

Figure 3. Sites and steps of activation of ER-alpha by nitrite. Six amino acids make up three sites that are involved in nitrite binding to ER-alpha. Lys529 and asn532 are labeled S1, his516 and lys520 makeup S2, and cys381 and his547 comprise S3.

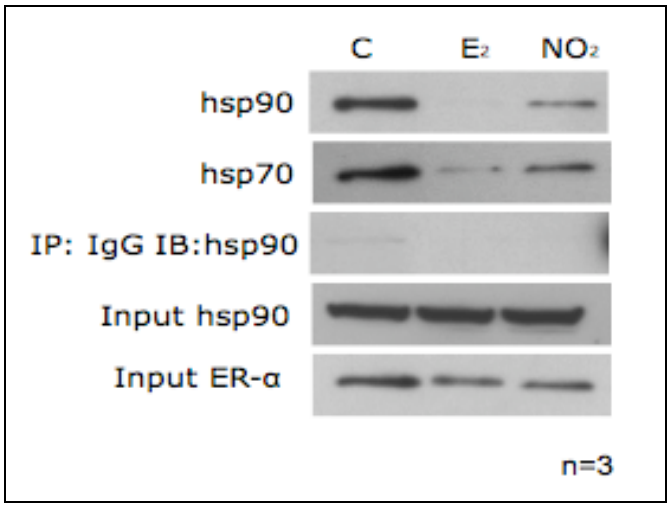

Figure 4. Estradiol and nitrite disrupt the interaction of ER-alpha with hsp90. HEK 293 cells were transfected with wild-type ER-alpha and treated for three hours with 1nM estradiol or 1uM sodium nitrite. Cells were immunoprecipitated with antibody for ER-alpha and western blot was performed to detect hsp90.

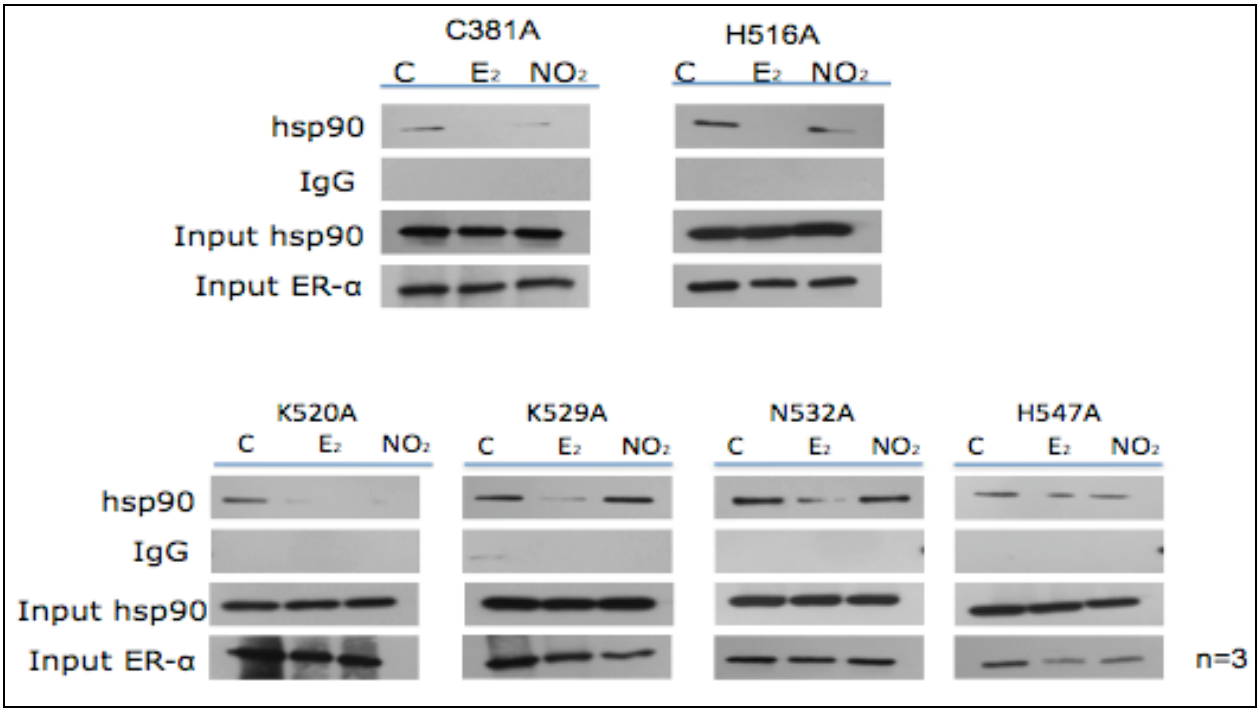

Figure 5. Interaction of mutant ER-alpha with hsp90 in the presence of estradiol or nitrite. Mutants forms of ER-alpha were transfected into HEK 293 cells, treated for three hours with 1nM estradiol or 1uM sodium nitrite and immunoprecipitated with antibody to ER-alpha followed by immunoblot.

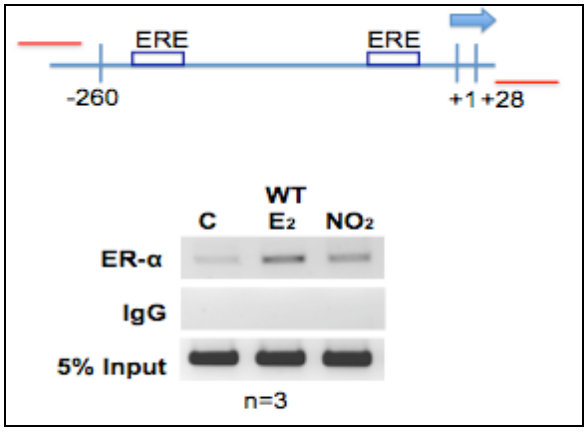

Figure 6. Recruitment of wild-type ER-alpha to the hC3 promoter. Following transfection with wild-type ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by reverse cross-linking. DNA was amplified using standard PCR and specific primers.

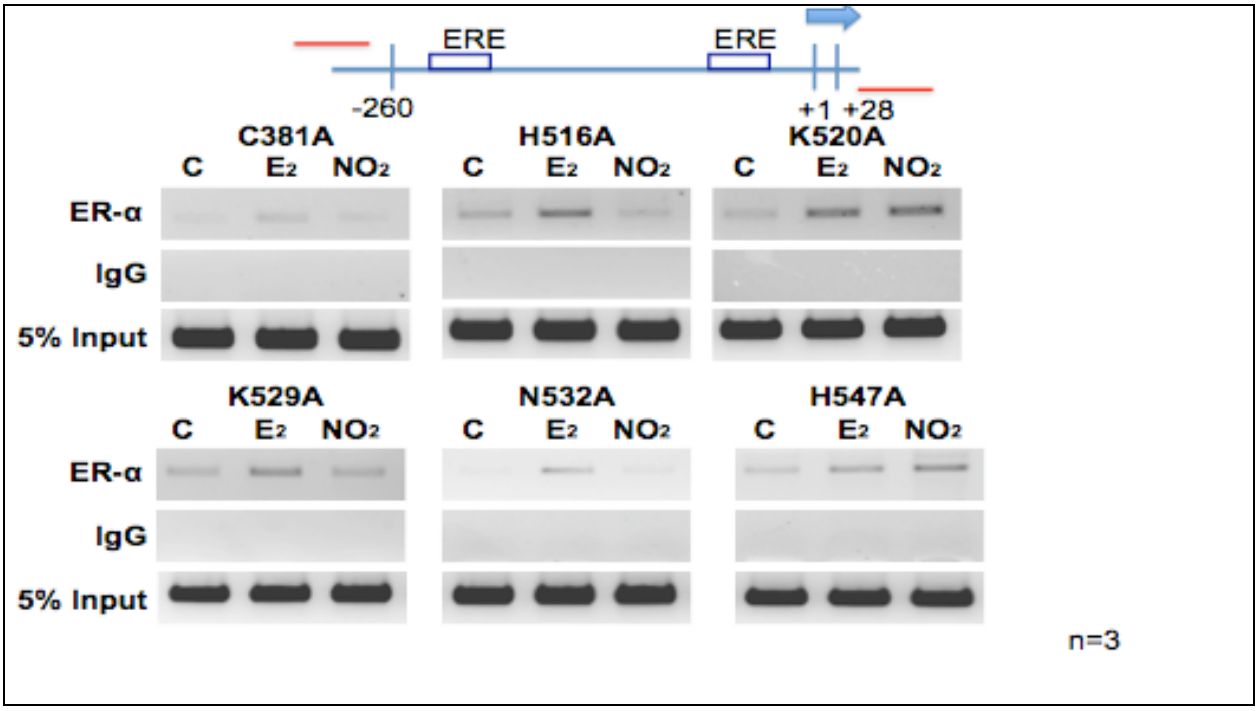

Figure 7. Recruitment of mutant ER-alpha to the hC3 promoter. Following transfection with mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by reverse cross-linking. DNA was amplified using standard PCR and specific primers.

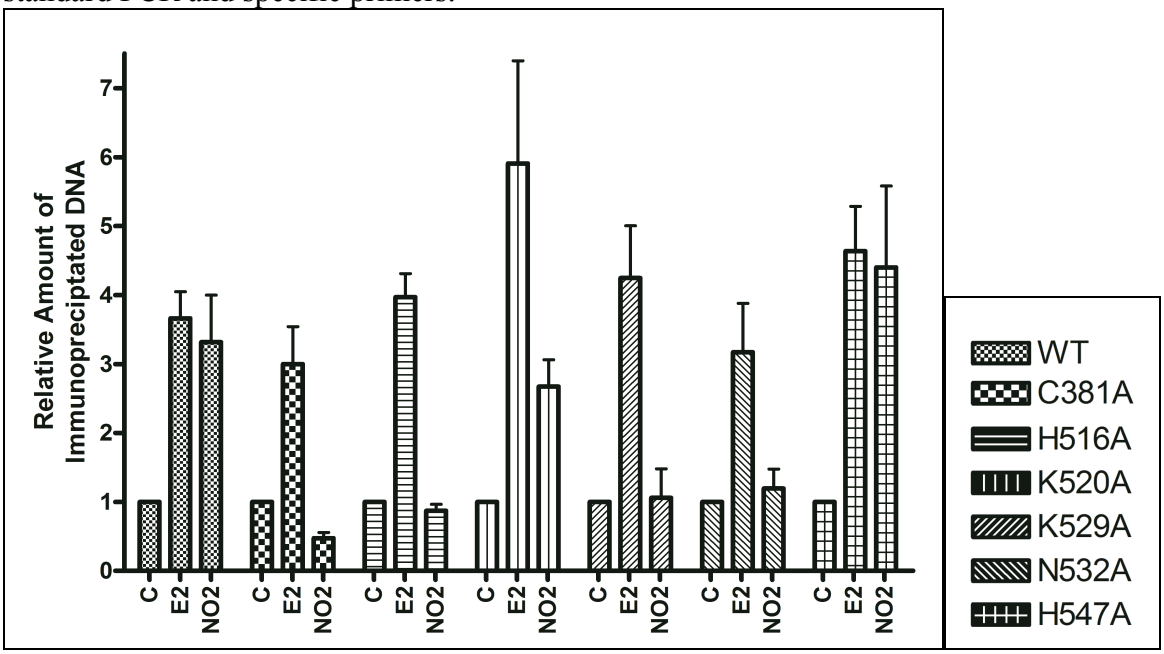

Figure 8. Quantitave analysis of wild-type and mutant ER-alpha recruitment to hC3 promoter. ChIP assays were performed and described above. Quantitative PCR was performed using SYBR green Values are expressed for each ER-alpha relative to corresponding control.

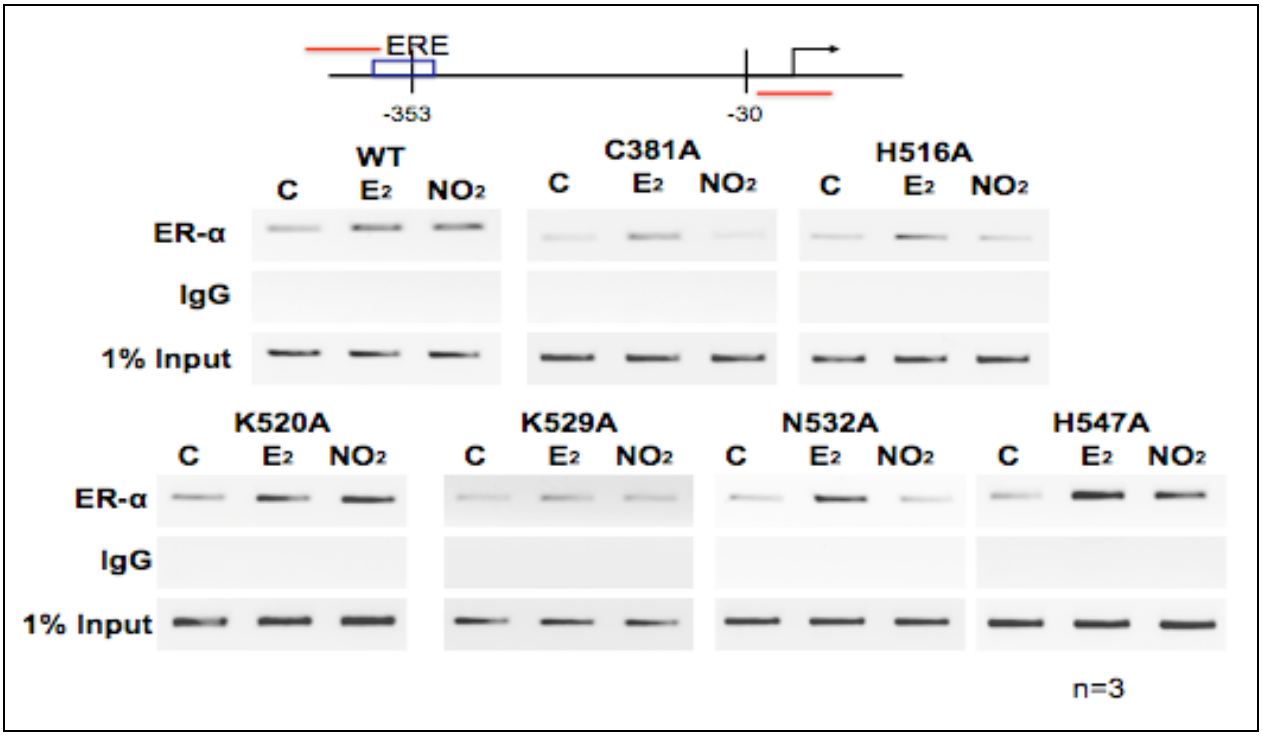

Figure 9. Recruitment of wild-type and mutant ER-alpha to the pS2 promoter. Following transfection with wild-type or mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by reverse cross-linking. DNA was amplified using standard PCR and specific primers.

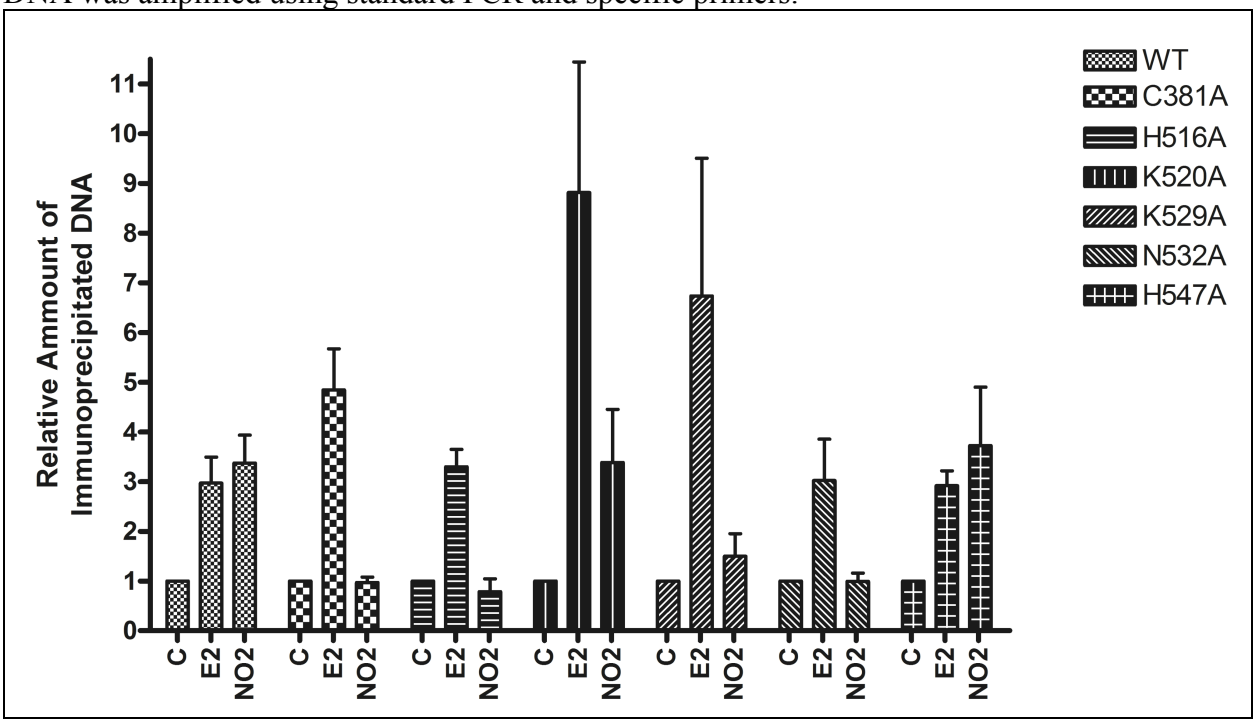

Figure 10. Quantitave analysis of wild-type and mutant ER-alpha recruitement to pS2 promoter. ChIP assays were performed and described above. Quantitative PCR was performed using SYBR green Values are expressed for each ER-alpha relative to corresponding control.

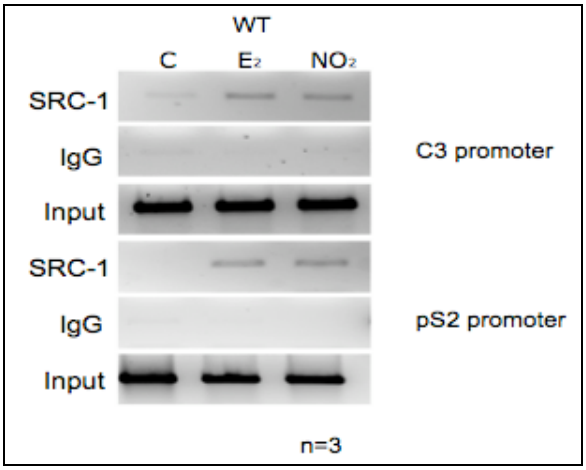

Figure 11. Recruitment of SRC-1 to the hC3 and pS2 promoter. Following transfection with wild-type ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to SRC-1. DNA was amplified using standard PCR and specific primers.

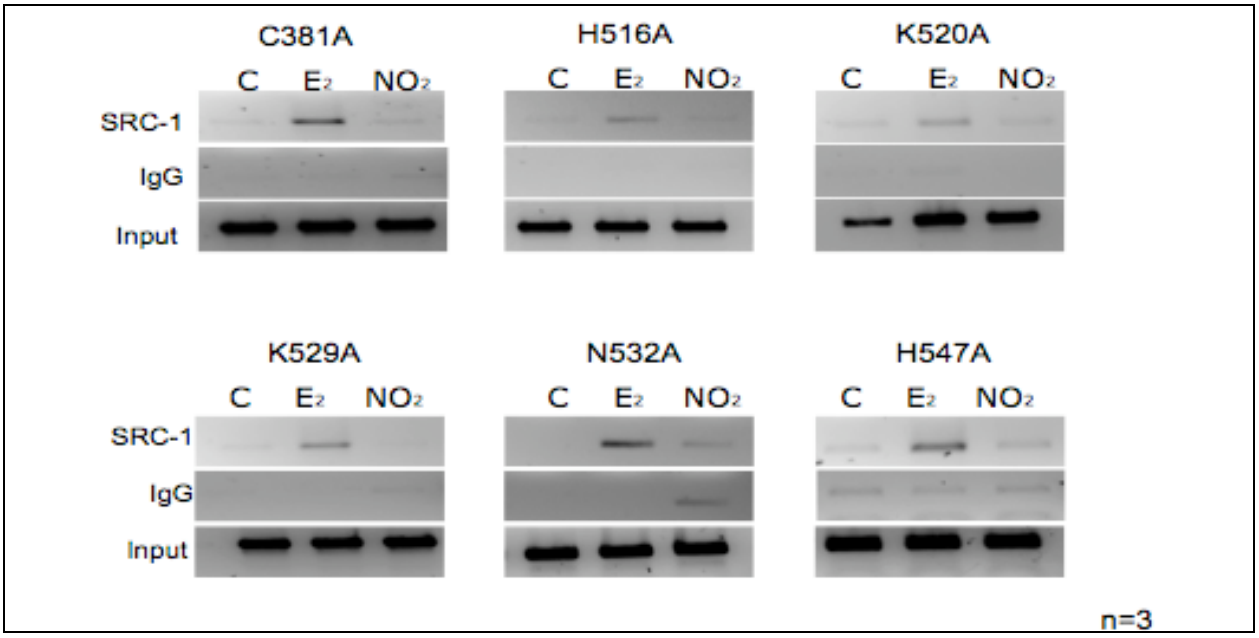

Figure 12. Recruitment of SRC-1 to the hC3 promoter. Following transfection with mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to SRC-1. DNA was amplified using standard PCR and specific primers.

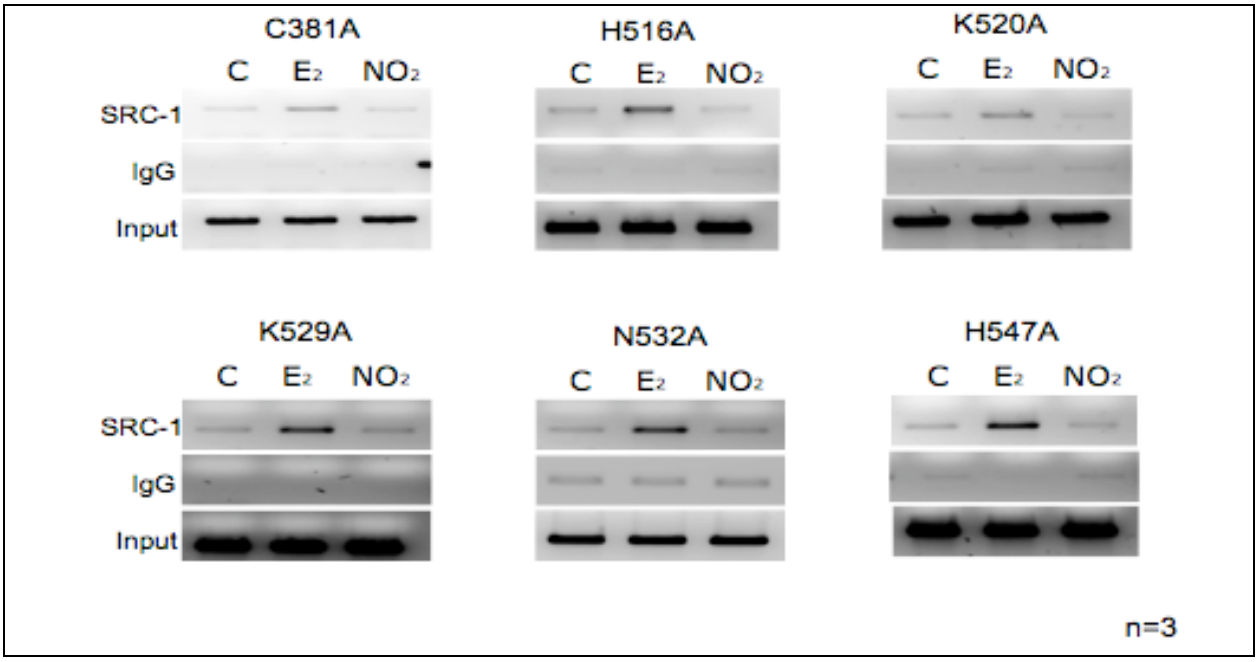

Figure 13. Recruitment of SRC-1 to the pS2 promoter. Following transfection with mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to SRC-1. DNA was amplified using standard PCR and specific primers.

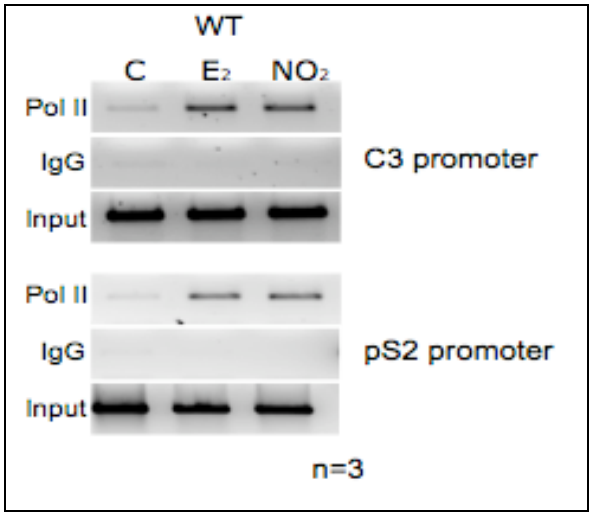

Figure 14. Recruitment of RNA Pol II to the hC3 promoter. Following transfection with wild-type ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to RNA Pol II. DNA was amplified using standard PCR and specific primers.

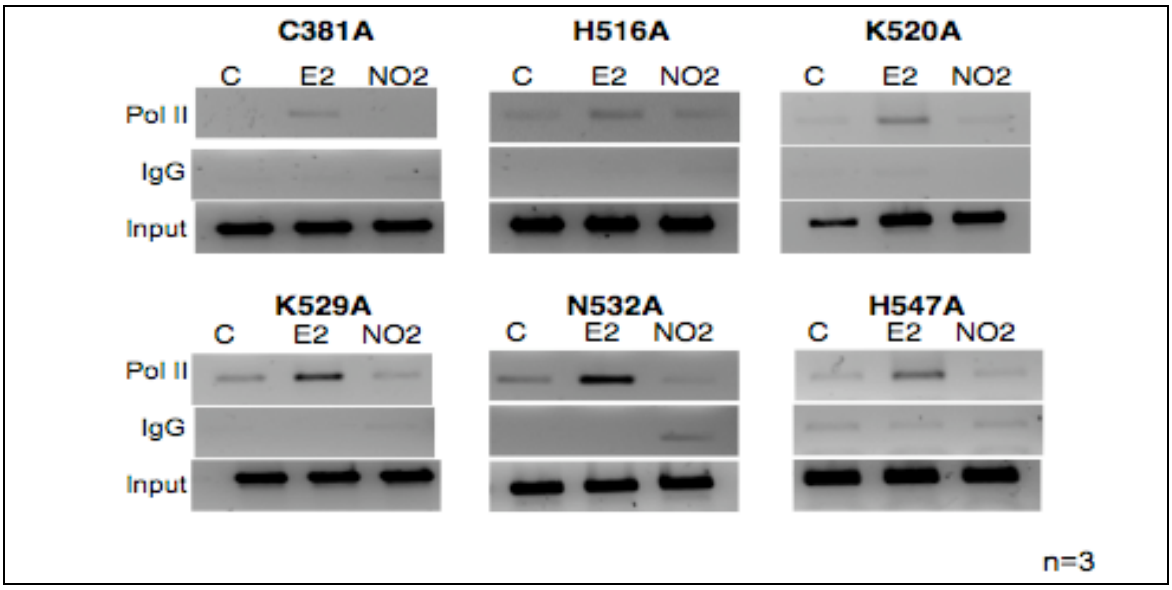

Figure 15. Recruitment of RNA Pol II to the hC3 promoter. Following transfection with mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to RNA Pol II. DNA was amplified using standard PCR and specific primers.

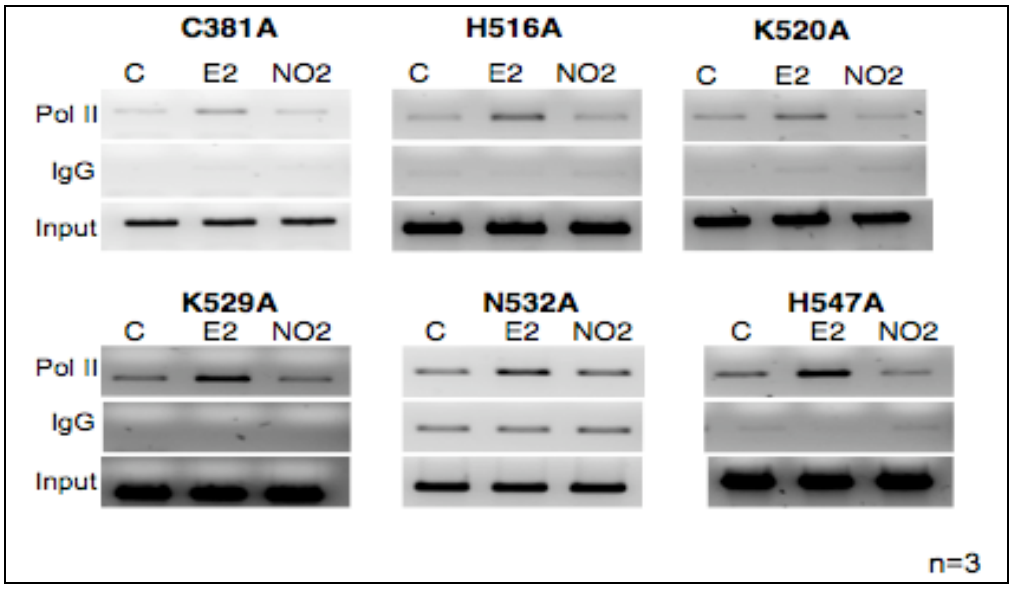

Figure 16. Recruitment of RNA Pol II to the pS2 promoter. Following transfection with mutant ER-alpha, HEK 293 were treated for three hours with 1nM estradiol or 1uM sodium nitrite, fixed with 1% formaldehyde, lysed and immunoprecipitated with antibody to ER-alpha. Antibody complexes were washed followed by a second immunoprecipitation with antibody to RNA Pol II. DNA was amplified using standard PCR and specific primers.